Survey and Isolation of Bacteriophages of Photosynthetic Purple Non-Sulfur and Marine Luminous Bacteria

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Microbial Diversity Course 1995

Abstract

A survey of the bacteriophages in fresh and marine waters of the Woods Hole, Mass. area was conducted. Twelve environmental isolates of purple non-sulfur bacteria, that likely included species of *Rhodobacter*, *Rhodopseudomonas*, *Rhodomicrobium* and *Rhodospirillum*, were used as hosts to identify plaque-forming units. Although several water sources were examined (School Street Marsh, Cedar Swamp, Lilly Pad Pond, etc.) no plaque forming phage were identified. Lysogenic strains were screened but they also were not found. However, in these latter studies, three potential phototrophic bacteriocin-producers were noted. Transmission electron microscopy (TEM) of one concentrated fresh water sample revealed the presence of several phage. Further studies are needed to identify productive phage-host pairs from these habitats, and to enhance the yield of plaque-forming phage. Three environmental isolates of luminous *Vibrio* (two probable *V. fischeri* and one *V. harveyi*) and one laboratory strain of *V. fischeri*, were used as hosts to survey sea water. Using the methods employed for screening fresh water, phage again were not obtained. Subsequently, a co-culture amplification procedure was successfully employed to yield a plaque-forming vibriophage and a probable bacteriocinogenic *Vibrio*. Preliminary host range characterization of the newly isolated phage, øMBL-1, was conducted.

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Introduction

Viruses of prokaryotes (e.g., bacteriophages) appear to occur in essentially all habitats occupied by the Bacteria or Archaea. Those environments for which the occurrence of bacteriophages has been surveyed include, but is not limited to, soils, marine and fresh waters, aerobic and anaerobic environs of multicellular Eukarya and the "extreme" environments frequently occupied by Archaea (e.g., hypersaline and thermophilic). The number of bacteriophages present in environmental samples appears to vary from very few to as many as 1E5 - 1E6 per ml of estuarine and pelagic marine waters. The vertebrate gastrointestinal tract contains numerous DNA and RNA phages per gram of feces.

Despite the prevalence of bacteriophages in ecologically significant environments, little is known, for example, of their influence on microbial population dynamics (e.g., "grazing"), intra- or inter-species gene transfer, or microbial evolution or adaptation. Although the occurrence, and often the morphology, of prokaryotic viruses from natural environments have been described, their molecular or genomic characterization is often lacking. The two-volume monograph *The Bacteriophages* (1) contains only two chapters focusing on non-coliphage viruses. In the several thousand page volume *The Prokayotes* (2), a scant two dozen genera are discussed with respect to isolated phages, most of which are only used for strain "typing". Therefore, a thorough characterization of virus-bacterial relationships from natural habitats can provide insights to the metabolic and genomic activities therein, and molecular characterization of prokaryotic viruses from diverse habitats can potentially yield new enzymes or molecules of biotechnological interest.

The objectives of this short-term project were to survey the occurrence, and potentially isolate, bacteriophages that infect bacteria of fresh and marine water habitats. The purple non-sulfur photosynthetic bacteria (3, 4) and the luminous marine *Vibrios* (5) were used as the fresh water and marine bacterial hosts. The presence of temperate bacteriophage in bacteria (lysogens) newly retrieved from the two environments was evaluated. In parallel determinations, the presence of lytic phage in the respective waters was examined by transmission electron microscopy.

Materials and Methods

Media. Photosynthetic purple non-sulfur bacteria were grown in liquid PNSB minimal medium (2); PNSB agar plating medium contained 1.5% 4X washed Difco agar. Complete medium (PYVS) consisted of 0.3% peptone, 0.5% soytone, and 0.3% yeast extract;

PYVS agar plating medium contained 1.5% agar. Soft top agar overlay media was as above, except the final agar concentration was 0.75 %. All reagents were from Difco.

Luminous bacteria were grown in sea water complete medium (SWC; 0.5% peptone, 0.5% tryptone, 0.5% yeast extract and 0.3 % glycerol. in 70% sea water). SWT medium contained 0.3% tryptone, 0.3% yeast extract and 0.3% glycerol in 70% sea water. Agar plating and soft top agar contained 1.5% and 0.75% Difco agar, respectively.

Mitomycin C (Sigma Chem., St. Louis, MO) was prepared as a sterile 0.2 mg/ ml dH_2O solution stored at 10°C in the dark.

Bacteria. Photosynthetic purple non-sulfur bacteria were isolated by course participants from a fresh water marsh (School Street Marsh; Woods Hole, MA) by vacuum filtration of 1 to 10 ml of water sample onto 47 mm 0.4 µm cellulose acetate disks. Disks were incubated on PNSB minimal agar medium in Gas-Pak H_2/CO_2 jars, illuminated with a 90 W GE tungsten lamp (8 - 12 inch distance), until colonies developed (approximately 4 days). Individual colonies were streaked two or more times onto PNSB agar and incubated as above until apparently axenic. Liquid PNSB cultures were obtained by inoculating 18 ml screw-cap tubes with isolated phototrophs, and incubating the closed tubes in the light for 2 - 4 days in PNSB.

Luminous marine bacteria were isolated on SWC agar medium by spreading 0.05 - 0.2 ml of water from surf (MBL Beach) or harbor (Eel Pond) sources, or by spreading a one loop volume of oyster gill (Garbage Beach; via Marine Resources Center) or gut material directly onto the agar surface. All samples were obtained in Woods Hole, MA. After approximately 15 hr at room temperature, luminous colonies were identified, transferred to SWC agar and streaked until apparently axenic. *Vibrio fischeri* (*ΔluxI*) was obtained from P. Dunlap, WHOI.

Bacteriophage plaque assays. *Lytic* bacteriophage were surveyed by mixing 0.05 - 0.1 ml of fresh or sea water samples with 0.05 - 0.2 ml of bacterial culture. Water samples were variably treated by filtration through glass fiber, 0.4 µm nylon polycarbonate, or concentration with 0.02 µm Acrodisks. For phototrophs, 2 day, turbid PNSB cultures were used as host cells. For luminescent bacteria, freshly-grown overnight, or 2 - 6 hr cultures (room temperature in SWT broth) were used. Where indicated, $CaCl_2$ and $MgSO_4$ were each added to the plating mixtures at 1 mM. After 15 min at rm. temp., cell/ water mixtures were added to 2.5 ml of molten top agar (48C) and distributed over the surface of PNSB or PYVS (phototrophs) or SWT (*Vibrios*) agar plates. Plates were incubated at rm temp for each cell type (as described above) and the presence of plaques evaluated over a 1 - 4 day period.

Prophage induction. *Temperate* bacteriophage were screened by incubating phototrophic or luminous bacteria in liquid PNSB or SWC medium, respectively, that contained 0.2 µg/ml Mitomycin C. Following 12 - 15 hr incubation (cell lysis was variably observed), one ml of the culture was clarified by microcentrifugation (twice) at full speed. Culture supernatants were used in plaque assays as described for lytic phage, or a one-loop volume was streaked onto the surface of an agar plate and then overlaid with top agar containing indicator cells. $CaCl_2$ and $MgSO_4$ were variably added to 1 mM.

Amplification of phage in sea water. 250 ml of sea water (collected from Eel Pond at the MBL dock adjacent to the Swope student center) was made up to SWT (6). 0.5 ml of mid-log phase, luminous *Vibrio* cultures (new isolates designated MBLB-1, Oys-1 and Oys-2 and a domesticated *V. fischeri* strain with a deletion in *luxI*; the latter obtained from P. Dunlap) were added to the SWT suspension. Following incubation overnight, 1/100 volume of $CHCl_3$ was added and the culture examined by the phage spot assay.

Phage purification. Phage were purified by removing an isolated plaque from the agar with a sterile Pasteur pipette, adding 50 µl of fresh cell culture in SWT, and incubating at rm temp for 15 min. 2.5 ml of SWT was added and the culture incubated at rm temp with aeration overnight. Chloroform (100 µl) was added to complete lysis.

Host range determination and spot assays. Rapid assays of lytic activity and host range were done by preparing a soft agar overlay (2.5 ml) of freshly grown *Vibrio* cultures (0.2 ml) on SWT. After the top agar had solidified, 10 µl of a culture supernatant or phage lysate was applied to the agar surface. Plates were incubated overnight, or longer, at rm temp.

Electron Microscopy. Concentrated (0.02 µm) water, clarified Mitomycin C-treated culture supernatants, or potential phage lysates, were applied (8 µl) to carbon-coated grids, wicked semi-dry with Kimwipes after ca. 4 min, and then negatively stained with 3% uranyl acetate or 2.5% ammonium molybdate solutions (8 µl) directly on the grids for 1 - 2 min. Air-dried grids were examined by TEM (Zeiss 10CA) in the MBL central microscopy facility.

Results and Discussion

Phage of Phototrophs

Lytic bacteriophages of purple non-sulfur bacteria were not isolated. A collection of 12 purple non-sulfur phototrophic bacteria isolated from a fresh water marsh was used to screen for bacteriophages in local fresh waters. Based on their cell morphology (light microscopy), pigmentation, motility (or lack thereof), variable heterotrophic growth in the dark with oxygen, and variable hybridization to rRNA probes for the α and B proteobacteria (see course exercises), the potential hosts likely included the genera *Rhodobacter, Rhodopseudomonas* and *Rhodomicrobium*. However, the strains were not definitively characterized to genus. In some experiments, the type strain *Rhodospirillum centenum* (C. Bauer, Indiana University) was used.

Water samples screened for plaque forming units were collected from the School Street Marsh, the ditch water near the ball field at the St. Joseph's Church Bell Tower playground (BFD), Cedar Swamp Marsh, and a private ornamental pond near the Gansett-Woods neighborhood. Water was used I) directly, ii) filtered through glass fiber, iii) filtered through 0.4 µm nylon membranes, or iv) concentrated 100-fold with 0.02 µm supported Acrodisk filters.

Attempts were made to isolate lytic phage directly from each of the water samples processed as described above (7, 8). Initially, 20 µl samples were spotted onto top agar lawns (PNSB or PYVS media) of the 12 phototrophic strains; the plates were then incubated

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anaerobically in the light. For all but three strains, the host bacteria failed to grow on PYVS agar; only PNSB was subsequently used. No zones of clearing or plaques were observed on any of these plates. The volume of water assayed was increased to 0.1 ml, and by 100-fold concentration, and in this case potential plaques were observed from the BFD water plated with phototrophs No. 8 and 12. One or more of these plaques were plucked and incubated 4 days with strain 8 or 12. Spot assays of these plaque enrichments failed to show plaques on several hosts tested (e.g., see Figure 1). In other assays that also failed to yield plaques, 1 mM $MgSO_4$ and $CaCl_2$ were added to the cell-water-top agar mixture during plating.

Apparent induction of bacteriocin by Mitomycin C. The presence of lysogenic phage in the collection of phototrophic bacteria was examined using Mitomycin C induction. Using 18 ml capped tubes, 2 ml of a culture grown in PNSB was transferred to the same medium, to which Mitomycin C was added at a final concentration of 0.2 μg/ml. After 3 - 5 days there was a marked reduction in the density of those cultures containing the radiomimetic agent compared to the untreated cultures. From the Mitomycin C cultures, 20 μl were applied in spot assays to lawns of several of the 12 phototrophs. Again, obvious plaques were not observed.

Apparent bacteriocin production was observed in the Mitomycin C incubations of phototrophs 1 and 3. The inhibitory activity appeared greatest on phototrophs 1, 3 and 8 (Figure 1). Although it could be argued that these strains are just highly sensitive to Mitomycin C, phototroph 8 was most sensitive to the induced cultures of strains 1 and 3, yet 8 grew fairly well from the induced culture.

Bacteriophage are present in local fresh water. Concentrated (0.02 μm) BFD water was negatively stained and examined for phage using TEM. In several grid fields examined, hexagonal (icosahedral?) phage head-like structures were observed (Figure 2). Occasionally, less organized negatively staining structures were observed that could be other forms of phage or virus particles.

Summary of phototroph survey. While phage abound in fresh waters (especially polluted, nutrient-rich water), the results obtained here suggest it is not always easy to find the suitable phage-host pair. No phage of the purple non-sulfur phototrophs were isolated. However, it does appear that bacteriocin production by some strains can be induced by Mitomycin C. These bactericidal compounds, often proteins, can be useful for characterizing cell populations, exploring gene regulation, or can be purified to exploit their specific enzymatic activity (e.g., RNase, DNase, membrane depolarization). It should be noted that the phototroph isolates 1 and 8 are brown pigmented, non-motile rods in clusters, and isolate 3 is a small motile rod; these bacteriocin producers may be members of the genera *Rhodobacter* or *Rhodopseudomonas*.

Future attempts at isolating phage of the phototrophs should utilize amplification methods employing large volumes of local water sources (see below and refs. 7 - 9). Alternatively, sewage can be screened, but it may not yield phage relevant to the generally unpolluted water bodies of the region. Nonetheless, such phage would be of potential genetic and ecological interest. Waterbury (10) has documented the natural adaptation of ocean cyanobacterial populations to phage resistance; resistance of co-occurring hosts to phage may account for the inability to detect plaque-forming phage for the natural Rhodospirillaceae isolates.

In one study (9) 16 different virulent bacteriophage types of *Rb. capsulata* were isolated. All were isolated from sewage and displayed differing plaque morphologies and host ranges. Although none of these lytic phages were able to carry out transduction, further reports on molecular biology or ecological significance of these phages is not apparent. However, other temperate phage have been isolated and more thoroughly characterized (11-13). Currently, the "gene transfer agent" (reviewed in 14 and 15) is the only phage-type gene transfer system (unidirectional) used in the Rhodospirillaceae, and apparently only in *Rb. capsulata*. Further characterization of new and previously isolated phages of the phototrophic purple non-sulfur bacteria appears warranted, particularly in light of the broad ecological distribution of these bacteria (3), and the complete genome analysis underway for *Rb. capsulata*. (16).

Phage of Luminous Vibrios

Direct screening of sea water did not yield vibriophages. Sea water samples (obtained almost exclusively from Eel Pond, Woods Hole) were treated by filtration essentially as described above for screening for viruses of phototrophs. Three environmental isolates of luminous *Vibrios* were used in lawns as indicator strains on SWC or SWT plating media. In spot or whole plate assays, no plaque forming units were detected. The presence of 1 mM $CaCl_2$ and $MgSO_4$ had no noticeable affect on cells nor did it result in phage detection. A somewhat larger volume of cell culture and water sample (0.5 ml of each), incubated at rm temp for 15 min prior to the addition of 2 ml of SWT top agar and plating, also failed to yield phage. Both Eel Pond and Cape Cod Bay sediment/water (from a depth of 20 m) were used. Concentrated (0.02 μm) Eel Pond sea water treated in this manner was also unproductive.

Freshly shucked whole oyster was homogenized with sterile SWC (20 g:40 ml) and the filtered (0.4 μm) homogenate spotted directly onto the environmental isolates. Alternatively, 0.1 ml of cells and homogenate were mixed, plated and again no plaques were observed.

Mitomycin C and heat shock failed to induce plaque forming phage. Three environmental *Vibrio* isolates were incubated overnight (fresh inocula) in 2 ml SWC containing Mitomycin C at 0.2 μg/ml. The cultures exhibited reduced growth or substantial clearing. When spotted onto SWC top agar lawns of all three *Vibrio* strains, no plaques or clearing was observed. A comparable experiment was conducted, again without interesting result, where fresh cultures were incubated at 50°C for 7 min, then 0.1 ml plated directly into top agar. Temperate prophage apparently were not induced, or they were unable to infect the three indicator strains.

The heat shock-treated culture of the oyster gut *Vibrio* (Oys-2) was filtered (0.4 μm), negatively stained and examined by TEM. Possible phage-like particles, spherical and lacking the head and tail structure typical of the Myoviridae were observed (data not shown).

Co-culture amplification of sea water yields vibriophage. The amplified Eel Pond sea water culture (see Materials and Methods) was spot assayed (40 μl) on lawns of the four *Vibrio* strains used for seeding. Plaques or growth inhibition was observed on the lawns of MBLB-1 and Oys-2 cells, but not on lawns of Oys-1 and *V. fischeri* (*ΔluxI*). Three

isolated plaques were purified from each plate as described above. Therefore, two plaque-forming phage appeared to be isolated on two separate *Vibrio* hosts.

Phage and bacteriocin host range. Although lysates of øMBL-1 could be titered (ca 5 x 10^8), Oys-2 lysates failed to yield plaques beyond a 1/10 dilution (Figure 3), suggesting bacteriocin, rather than phage, was responsible for the turbid "plaque" morphology. Therefore, cells from the centers of cleared zones were streaked; isolated colonies were then picked onto lawns of the four *Vibrio* strains. Figure 4 (bottom two rows) shows the small halo of clearing surrounding the colonies derived from the "øOys-2 plaques" when these cells are placed on a lawn of Oys-2 cells. Three culture supernatants of "øOys-2 lysates" (second row) inhibit Oys-2 growth. Therefore, bacteriocin production, active only against Oys-2 cells, has been detected. øMBL-1 clears the MBLB *Vibrio* strain significantly, and causes turbid clearing of Oys-2. It does not appear to infect the other two vibrios. Colonies from the centers of plaques do not produce halos of clearing on any of the indicator strains. Therefore these appear to be bonafide phage-resistant, non-bacteriocin producing derivatives.

TEM reveals phage-like particles without elaborate tails. øMBL-1 lysates that were untreated, filtered (0.4 µm), or incubated with MBLB *Vibrio* cells were negatively stained and examined by TEM. Phage head-like particles were apparent, but clear, elaborate tail structures were absent (data not shown). Although preparation of this marine vibriophage for TEM has not been optimized, the particles observed suggest the phage is a member of the viral family *Podoviridae*.

Summary of vibriophage isolation. Isolation of a few phages of the luminous *Vibrios* has been reported (6, 17), with progress toward developing a system to transduce genes among *V. fischeri* strains (6). Characterization of additional phage will enhance the genetic analysis of *Vibrio*, but are worthy of study in their own right. Gene regulatory mechanisms have not been extensively studied in the marine vibrios and the vibriophages should have something to contribute to that area. The population dynamics of luminous vibrios, including "quorum sensing", particularly as it relates to cell surfaces and receptors (18), can be addressed using phage as a biological probe. Recent studies on colonization by luminous symbionts suggest that phage may be helpful in characterizing relevant surface components of the participating *Vibrios* (19; K. Viseck, pers. comm.).

Amplification by seeded cultures, using more than one strain as an inoculum, was shown to be a useful approach for isolating vibriophages. In this single example, more than one "bacteriolytic" activity was identified, so potentially multiple phage can be enriched in a single amplification culture. In less than two days, at least one lytic phage, øMBL-1, was isolated by the simple seeded amplification procedure.

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Figure 1. Bacteriocin production by three purple non-sulfur phototrophs treated with Mitomycin C.

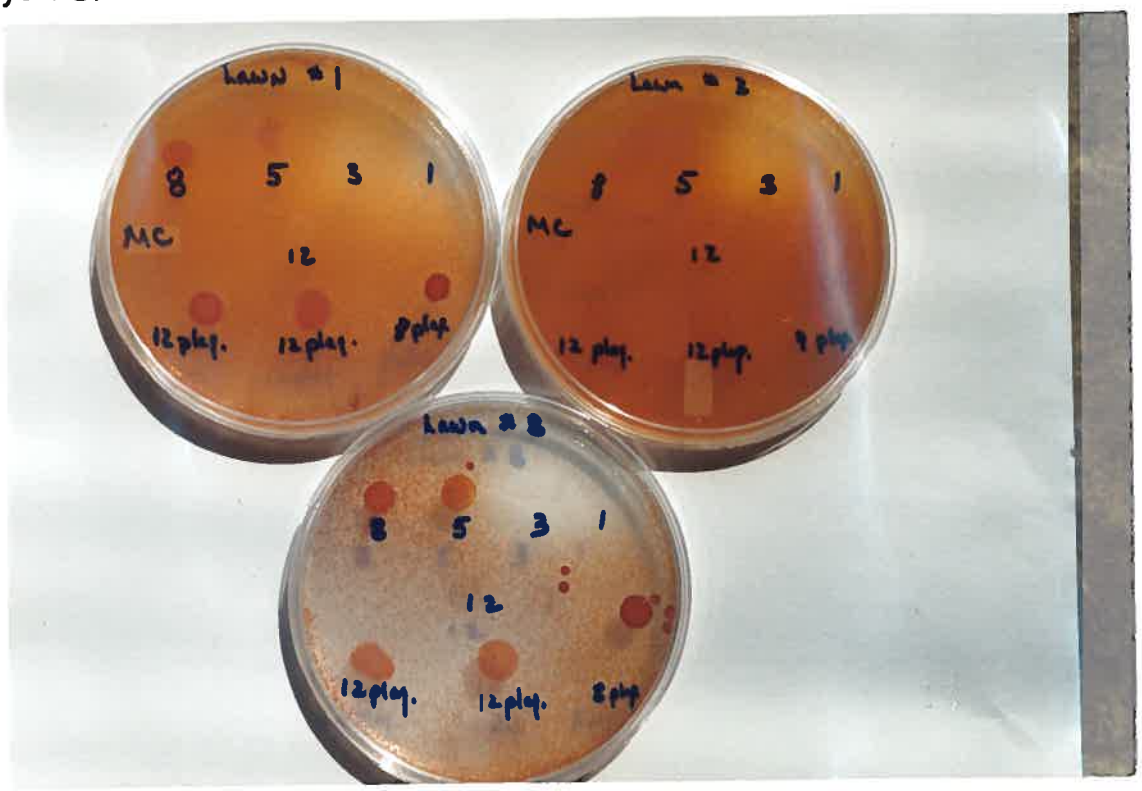

Figure 2. TEM of negatively stained phage particles from ditch water (BFD). 50K and 100K magnification of two different particles, with a 2X photographic enlargement.

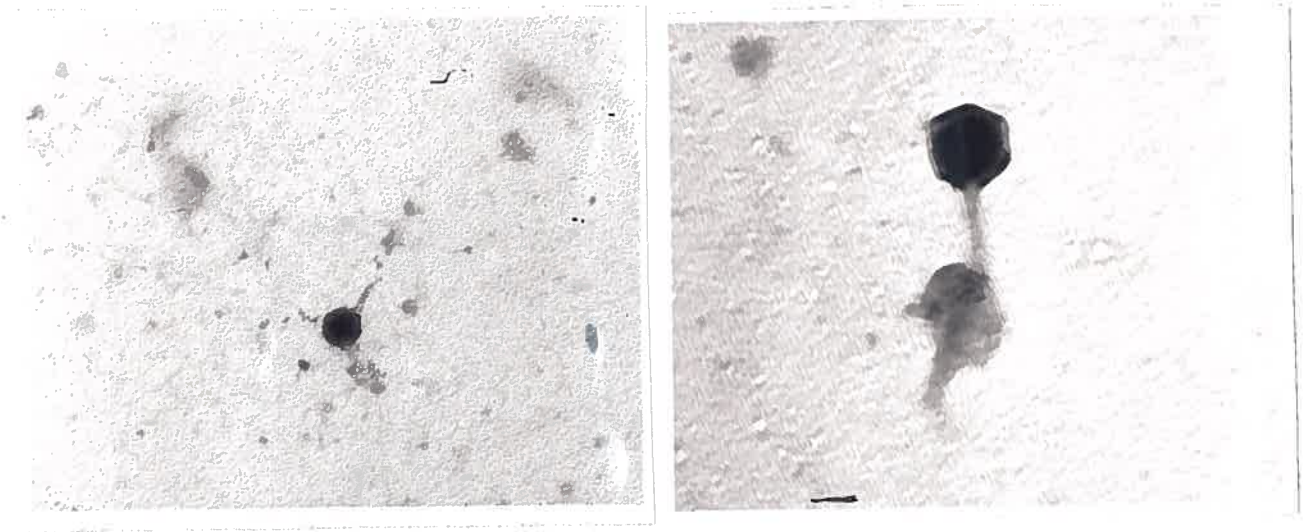

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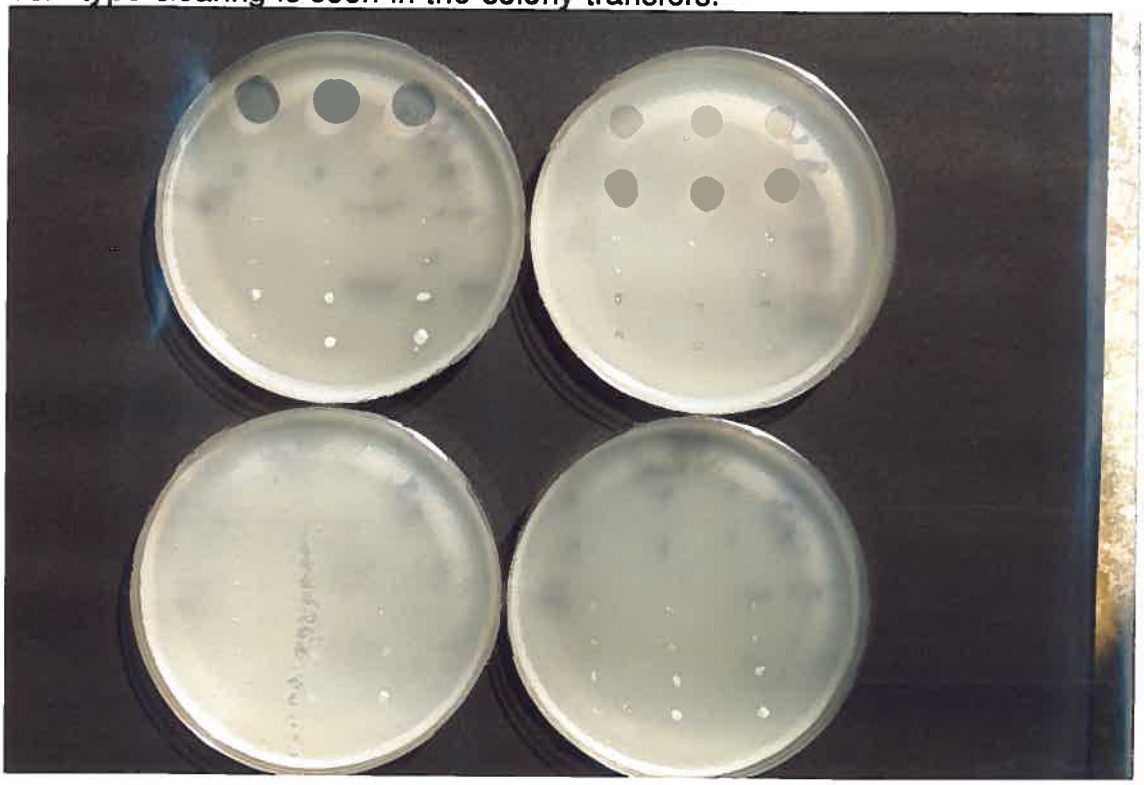
Figure 3. Titers of lytic activities. Top row is a lysate of øMBL-1 (prepared on strain MBLB-1) diluted to 10^{-7} and 10^{-8} and plated on MBLB cells. The bottom row is a "lysate of øOys-2" (prepared on Oys-2 cells) diluted 10^{-1} and 10^{-2} and plated on Oys-2 cells. Phage and bacteriocin activity are apparent.

Figure 4. Host range of phage and bacteriocin activities. Cell lawns are: *top* row, MBLB, Oys-2 (l to r); *bottom* row, Oys-1 and *V. fischeri*. For lysate spot assays, the top row on each plate is 3 separate lysates of øMBL-1 and the second row is 3 separate preparations of "øOys-2". Below the phage are colony transfers of potential lysogens or resistant cells from lysis zones of øMBL-1 (two rows) and "øOys-2" (bottom two rows). Note that øMBL-1 has a broader host range and substantially clear MBLB cells. øOys-2 affects only Oys-2 cells, and bacteriocin-type clearing is seen in the colony transfers.



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